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Involvement of protein kinase C in the presynaptic nicotinic modulation of [^{3}H]-dopamine release from rat striatal synaptosomes

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1 Presynaptic nicotinic ACh receptors modulate transmitter release in the brain. Here we report their interactions with protein kinase C (PKC) with respect to [^{3}H]-dopamine release from rat striatal synaptosomes, monitored by superfusion.

2 Two specific PKC inhibitors, Ro 31-8220 (1 μM) and D-*erythro*-sphingosine (10 μM) significantly reduced (by 51 and 26% respectively) [^{3}H]-dopamine release stimulated by anatoxin-a (AnTx), a potent and selective agonist of nicotinic ACh receptors. The inactive structural analogue of Ro 31-8220, bisindolylmaleimide V (1 μM) had no effect.

3 Two phorbol esters, PDBu (1 μM) and PMA (1 μM) potentiated AnTx-evoked [^{3}H]-dopamine release by 50–80%. This was Ca^{2+}-dependent and prevented by PKC inhibitors. In the absence of nicotinic agonist, phorbol esters enhanced basal release through a PKC-independent mechanism.

4 A $^{86}Rb^+$ efflux assay of nicotinic ACh receptor function confirmed that Ro 31-8220 has no nonspecific effect on presynaptic nicotinic ACh receptors.

5 These results suggest that PKC is activated by nicotinic ACh receptor stimulation and mediates a component of AnTx-evoked [^{3}H]-dopamine release. In addition, independent activation of PKC can further amplify the response, offering a potential mechanism for receptor crosstalk.
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Keywords: Presynaptic nicotinic acetylcholine receptors; rat striatal synaptosomes; dopamine release; protein kinase C; phorbol esters

Abbreviations: ACh, acetylcholine; AnTx, (±)anatoxin-a; DMSO, dimethyl sulphoxide; EGTA, ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic acid; GAP-43, growth-associated protein; PDBu, phorbol-12,13-dibutyrate; 4α-PDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; Ro 31-8220, 3-[1-[3(amidinothio)propyl-1H-indol-3-(1-methyl-1H-indol-3-yl)maleimide methane sulphonate

Introduction

Nicotinic acetylcholine (ACh) receptors are widely expressed in the brain. These pentameric ligand-gated cation channels are comprised of one or more types of subunit from a portfolio of nine homologous subunits expressed by mammalian CNS neurones (α2–α7; β2–β4). Despite this potential for heterogeneity of nicotinic ACh receptors, their physiological role in the brain is far from clear, with few examples of nicotinic transmission at central synapses (Role & Berg, 1996; Jones *et al*., 1999). However, the presence of presynaptic nicotinic ACh receptors able to facilitate the release of various transmitters is well documented (Wonnacott, 1997; Kaiser *et al*., 2000), and a primary role of nicotinic ACh receptors in the brain may be to 'modify rather than mediate' synaptic transmission (Role & Berg, 1996).

A well-studied example of presynaptic nicotinic ACh receptor function is the modulation of dopamine release from striatal nerve terminals. In superfused synaptosome preparations nicotinic agonists elicit [^{3}H]-dopamine release in a dose-dependent manner and this response is blocked by nicotinic antagonists such as mecamylamine, dihydro-β-erythroidine and chlorisondamine (Grady *et al*., 1992; El Bizri & Clarke, 1994; Soliakov *et al*., 1995). More recently, studies with the α3β2-selective antagonist α-conotoxin MII (Kulak *et al*., 1997; Kaiser *et al*., 1998) and the novel agonist UB-165 (Sharples *et al*., 2000) support the involvement of both α3β2 and α4β2 containing nicotinic ACh receptors in the presynaptic modulation of [^{3}H]-dopamine release from striatal synaptosomes. Neuronal nicotinic ACh receptors have high relative permeability to Ca^{2+} and Ca^{2+} entry accompanying receptor activation might be sufficient to stimulate exocytosis directly. However, [^{3}H]-dopamine release elicited by nicotinic agonists is both Na^+ and Ca^{2+} dependent (El Bizri & Clarke, 1994; Soliakov *et al*., 1995), and largely blocked by ω-conotoxin GVIA, implicating N-type Ca^{2+} channels in the mechanism (Soliakov & Wonnacott, 1996). Thus in this *in vitro* preparation agonists acting at presynaptic nicotinic ACh receptors appear to cause dopamine release by local depolarization and activation of voltage operated Ca^{2+} channels. Nevertheless, the transient influx of Ca^{2+} through the nicotinic channel could link nicotinic ACh receptor activation to second messenger pathways, enabling these receptors to exert more subtle modulatory roles than simply triggering transmitter release. One Ca^{2+}-dependent candidate is protein kinase C (PKC); its involvement in transmitter release processes is well docu-

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mented (Malenka *et al*., 1986; Dekker *et al*., 1991) although there is debate about the underlying mechanisms (see Waters & Smith, 2000).

Evidence for an interaction between nicotinic ACh receptor stimulation, PKC activation and increased exocytosis has come from studies on adrenal medullary cells (Wakade *et al*., 1986; TerBush *et al*., 1988; Cox & Parsons, 1997). In these cells, the nicotine-induced secretion of catecholamines is associated with the entry of extracellular Ca^{2+} (Wakade *et al*., 1986), rapid translocation of PKC from cytosol to membrane (TerBush *et al*., 1988) and the concomitant activation of PKC (Brocklehurst *et al*., 1985). It has been proposed that PKC enhances exocytosis by increasing the size of the readily releasable pool of transmitter in chromaffin cells (Gillis *et al*., 1996).

In the present studies, we have used activators and specific inhibitors of PKC to dissect the contribution of PKC to nicotinic ACh receptor-stimulated [^{3}H]-dopamine release from rat striatal synaptosomes. The results show that PKC accounts for part of the nicotinic response. In addition, the nicotinic response is enhanced by independent activation of PKC.

Methods

Superfusion of rat striatal synaptosomes for [^{3}H]-dopamine release

Preparation of P2 synaptosomes and basic superfusion protocols were carried out as previously described (Soliakov *et al*., 1995; Soliakov & Wonnacott, 1996). Briefly, rats were killed by cervical dislocation, striata dissected and immediately used for the preparation of synaptosomes. The washed P2 pellet was resuspended in Krebs-bicarbonate buffer ($\sim$5 mg of protein ml^{-1}, determined using the method of Lowry *et al*., 1951) of the following composition (in mM): NaCl 118, KCl 2.4, $CaCl_2$ 2.4, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10, previously bubbled for 1.5 h with 95% O_2/5% CO_2 gas mixture and adjusted to pH 7.4. To prevent degradation of dopamine, the buffer was supplemented with ascorbic acid (1 mM) and pargyline (8 μM). The dopamine uptake inhibitor nomifensine (0.5 μM) was added subsequently to block transmitter reuptake during the superfusion procedure. After loading with [^{3}H]-dopamine (0.185 MBq ml^{-1}, 15 min at 37°C), synaptosomes were placed into superfusion chambers in a modified Brandel apparatus. Superfusion was carried out as described earlier (Soliakov *et al*., 1995), except that only one stimulation (40 s) with AnTx (1 μM) was given after a 30 min wash out period. The agonist pulse was separated from the bulk flow of the buffer by 10 s air bubbles. In some controls, agonist was replaced with a 40 s buffer pulse. Fractions (2 min) were collected and counted for radioactivity in a Packard scintillation spectrometer (counting efficiency 48%). In a previous study (Rapier *et al*., 1988), we have confirmed that the radioactivity released under these conditions corresponds to [^{3}H]-dopamine.

PKC inhibitors, or mecamylamine were applied to the superfusion medium 10 min before stimulation and maintained until experiments were completed. Phorbol esters were present in the Krebs-bicarbonate buffer for 2 min before stimulation only, as illustrated in Figure 3A. In Ca^{2+}-free medium, $CaCl_2$ was substituted by an equimolar concentration of $MgCl_2$ and the buffer was supplemented with 5 mM EGTA. In Ca^{2+}-free experiments, synaptosomes were prepared and loaded with [^{3}H]-dopamine in Krebs-bicarbonate buffer without Ca^{2+}. The preparation was then divided and one half was superfused in normal superfusion medium while the other was maintained in Ca^{2+}-free conditions. To study the Ca^{2+}-dependence of the effects of phorbol esters on spontaneous [^{3}H]-dopamine release, phorbol esters were applied during the last 2 min of the wash out period and the 40 s stimulus consisted of normal or Ca^{2+}-free buffer instead of agonist or depolarizing stimulus. Perfusion with the same medium was then continued until the experiment was completed.

Monitoring of $^{86}Rb^+$ efflux from superfused rat thalamus synaptosomes

$^{86}Rb^+$ efflux from superfused synaptosomes was measured essentially as described previously (Marks *et al*., 1996; Sharples *et al*., 2000). Briefly, rat thalamus synaptosomes were prepared by differential centrifugation in accordance with Soliakov *et al*. (1995). Loading of synaptosomes with $^{86}Rb^+$ ($\sim$70 MBq per chamber) was carried out at 22°C for 30 min. After loading, synaptosomes were placed on GF/C filters and inserted into open superfusion chambers (Soliakov *et al*., 1995), and samples were perfused at 2.5 ml min^{-1} with superfusion buffer (mM): NaCl 135, KCl 1.5, $CaCl_2$ 2.0, $MgSO_4$ 1.0, glucose 20, HEPES 25, pH 7.5, supplemented with BSA (0.1% w v^{-1}), CsCl (5 mM) and tetrodotoxin (50 nM). After a 6 min washout period, stimulation with AnTx (1 μM) in the presence or absence of inhibitors was applied for 1 min. Fractions (30 s) were collected and counted for radioactivity in a Packard scintillation spectrometer.

Data analysis

[^{3}H]-Dopamine release and $^{86}Rb^+$ efflux were calculated as the area under the peak of release above baseline. The baseline for [^{3}H]-dopamine release and $^{86}Rb^+$ efflux from superfused synaptosomes was derived by fitting double exponential decay equation 1 to the experimental data, using the SigmaPlot for Windows software:

$$y = ae^{-bx} + ce^{-dx} \quad (1)$$

where a, b, c, and d are the curve parameters and x is the fraction number. In most cases evoked [^{3}H]-dopamine release was calculated as the amount of radioactivity released above baseline and presented as a percentage of total radioactivity in synaptosomes at the moment of stimulation (fractional release) and then normalized by expressing them as a percentage of the corresponding control; the control (AnTx-evoked [^{3}H]-dopamine release in the absence of other drugs or treatments) serves as an internal standard and facilitates averaging data from independent experiments. In experiments comparing normal and Ca^{2+}-free conditions (Figure 4), fractional release was not computed because of the different levels of basal release under these conditions (which influences the residual radioactivity in synaptosomes at the

moment of stimulation). In this case, released [^{3}H]-dopamine is calculated as fmol mg^{-1} of synaptosomal protein. Agonist-evoked $^{86}Rb^+$ efflux was calculated as the fractional release above base line.

Values are the mean$\pm$s.e.mean of the number of experiments indicated, each consisting of two or three replicate chambers for each condition. Statistical analysis of differences from control was performed using the Student's paired *t*-test or one-way ANOVA. In all cases, $P<0.05$ was considered statistically significant.

Materials

Male Sprague-Dawley rats (average weight 250 g) were obtained from Bath University Animal House breeding colony. [7,8-^{3}H]-dopamine (specific activity 1.78×10^{12} Bq mmol^{-1}) was purchased from Amersham International (Amersham, Bucks, U.K.). ^{86}RbCl (specific activity $>3.7\times10^{10}$ Bq g^{-1}) was obtained from NEN Life Science Products (Hounslow, U.K.). PKC inhibitors D-*erythro*-sphingosine (free base), Ro 31-8220, the inactive analogue bisindolylmaleimide V, and phorbol esters phorbol-12,13-dibutyrate (PDBu), phorbol-12-myristate-13-acetate (PMA) and 4α-phorbol-12,13-didecanoate (4α-PDD) were purchased from Calbiochem (Nottingham, U.K.). All phorbol esters were stored for up to 2 months at -20°C as a 2–5 mM stock in DMSO. ($\pm$)Anatoxin-a (AnTx) was from Tocris Cookson (Bristol, U.K.). Mecamylamine, pargyline and nomifensine were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). All other chemicals used were of analytical grade and obtained from standard commercial sources.

Results

Effects of PKC inhibitors on AnTx-evoked [^{3}H]-dopamine release

[^{3}H]-Dopamine release from striatal synaptosomes was evoked by a 40 s application of the potent and specific nicotinic agonist ($\pm$)anatoxin-a (AnTx, Figure 1A), as previously demonstrated (Soliakov *et al*., 1995; Soliakov & Wonnacott, 1996). To determine if PKC contributes to AnTx-evoked [^{3}H]-dopamine release, the effect of PKC inhibitors was examined. Synaptosomes were exposed to drugs for 10 min prior to stimulation with AnTx. Ro 31-8220 (1 μM) had no effect on basal release but significantly decreased AnTx-evoked [^{3}H]-dopamine release by 33.5$\pm$4.6% ($P<0.01$, $n=8$; Figure 1A,B). This concentration of Ro 31-8220 should fully inhibit PKC ($IC_{50}=10$ nM; Davis *et al*., 1992a), while retaining specificity for PKC. Investigation of the timecourse of this inhibition showed that the maximum inhibition by Ro 31-8220 was achieved after 7 min preincubation (Figure 1C). Another, structurally unrelated, PKC antagonist, D-*erythro*-sphingosine (free base, 10 μM) produced a smaller but statistically significant decrease in AnTx-evoked [^{3}H]-dopamine release of 19.1$\pm$3.3% ($P<0.01$, $n=6$; Figure 1B). This inhibitor is less potent than Ro 31-8220 ($IC_{50}=2.8$ μM; Merrill *et al*., 1989); but higher concentrations could not be tested because of its limited solubility in Krebs-bicarbonate buffer. In contrast, the inactive structural analogue of Ro 31-8220, bisindolylmaleimide V (1 μM; Davis *et al*., 1992b) was without effect on AnTx-evoked [^{3}H]-dopamine release, as was the vehicle DMSO (Figure 1B). The effects of these inhibitors on the specific, nicotinic ACh receptor-mediated response to AnTx, defined by the antagonist mecamylamine, are tabulated in Table 1.

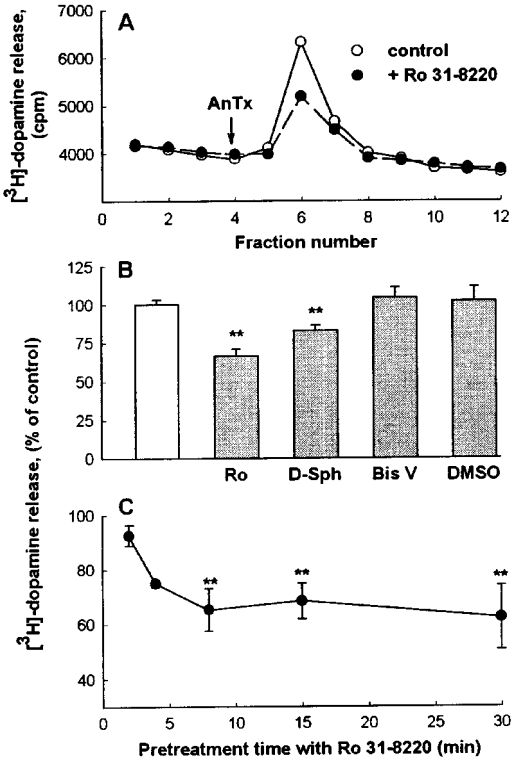

Figure 1 Effects of PKC inhibitors on AnTx-evoked [^{3}H]-dopamine release from rat striatal synaptosomes. (A) Typical profiles for [^{3}H]-dopamine release from superfused striatal synaptosomes in normal Krebs-bicarbonate buffer (control) or in the presence of Ro 31-8220 (1 μM). Inhibitor was introduced into the buffer 10 min prior to stimulation with 1 μM AnTx (40 s, arrow) and maintained in the superfusion medium until the experiment was completed. Fractions were collected at 2 min intervals and counted for released [^{3}H]-dopamine. (B) Synaptosomes were superfused with Krebs-bicarbonate buffer, containing Ro 31-8220 (Ro, 1 μM), D-*erythro*-sphingosine (D-Sph, 10 μM), bisindolylmaleimide V (Bis V, 1 μM) or vehicle (DMSO, 0.05%) for 10 min before stimulation with 1 μM AnTx for 40 s, as in (A). Release of [^{3}H]-dopamine above baseline in the presence of drugs was calculated as a per cent of the AnTx-evoked release determined in parallel in controls not exposed to drug. Values are the mean$\pm$s.e.mean of 6–8 independent experiments. (C) Time course for inhibition of AnTx-evoked [^{3}H]-dopamine release from synaptosomes by Ro 31-8220 (1 μM). This inhibitor was applied to the Krebs-bicarbonate buffer for the time period indicated, before stimulation with AnTx (1 μM, 40 s). Values are the mean$\pm$s.e.mean of five independent experiments. **Significantly different from control, $P<0.01$, Student's paired *t*-test.

To ensure that the effects of Ro 31-8220 on AnTx-evoked [^{3}H]-dopamine release were not due to a nonspecific action on the nicotinic ACh receptor itself, its effect on AnTx-evoked $^{86}Rb^+$ efflux from superfused synaptosomes was examined. AnTx elicited $^{86}Rb^+$ efflux, which is attributed to a direct flux through the nicotinic ACh receptor channel (Marks *et al*., 1996). As shown in Figure 2, addition of Ro 31-8220 (1 μM) to the superfusion medium did not modify $^{86}Rb^+$ efflux from synaptosomes stimulated with AnTx (1 μM, 1 min). In

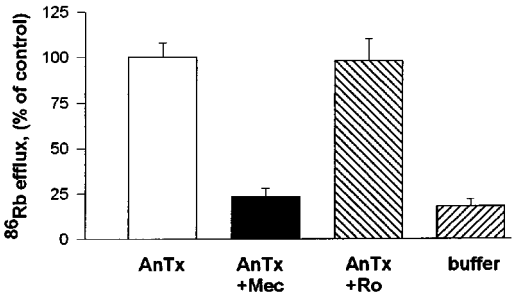

Figure 2 Effects of drugs on $^{86}Rb^+$ efflux from synaptosomes: an assay of nicotinic ACh receptor function. Rat thalamic synaptosomes were loaded with ^{86}RbCl as described in Methods. Synaptosomes were prepared from thalamus as this brain region gives the biggest signal, necessary for a quantitative assay (Marks *et al*., 1996). After a 6 min washout, synaptosomes were stimulated with AnTx (1 μM, 1 min) either alone (AnTx, control) or in the presence of mecamylamine (Mec; 10 μM) or Ro 31-8220 (Ro; 1 μM). Both inhibitors were introduced into the perfusing buffer 10 min before the AnTx pulse and were maintained in the medium until the experiment was completed. Parallel chambers were challenged with a buffer pulse for comparison. Fractions (30 s) were collected and counted for radioactivity. Values are the mean$\pm$s.e.mean of 4–5 experiments.

contrast, the nicotinic antagonist mecamylamine (10 μM) reduced AnTx-evoked $^{86}Rb^+$ efflux to the same level as a buffer pulse alone (Figure 2). Taken together, these results suggest that a part of nicotinic ACh receptor-evoked [^{3}H]-dopamine release from rat striatal synaptosomes is realised through the involvement of PKC.

Effects of PKC activators on AnTx-evoked [^{3}H]-dopamine release

Having determined that PKC inhibitors can attenuate AnTx-evoked [^{3}H]-dopamine release, we next examined the effect of an activator of PKC, the phorbol ester PDBu. In the absence of stimulation by AnTx, PDBu (applied for 2 min prior to a buffer pulse instead of nicotinic agonist) itself enhanced the basal release of [^{3}H]-dopamine from striatal synaptosomes (Figure 3A,B). A similar response was seen to PMA (Figure 4A). Pretreatment of synaptosomes for 2 min with PDBu produced a concentration-dependent enhancement of subsequent AnTx-evoked [^{3}H]-dopamine release (data not shown). This effect was maximal at 1 μM PDBu, with an increase in release of radiolabel to 212.3$\pm$19.7% of control ($P<0.001$, $n=5$, Figure 3B). The inactive analogue 4α-PDD, examined over a similar concentration range as PDBu, had no significant effect compared with control (Figure 3B).

To distinguish any effect of PDBu on the nicotinic component of evoked release, mecamylamine was used to block the receptor-mediated response elicited by AnTx. Mecamylamine (10 μM) decreased AnTx-evoked [^{3}H]-dopamine release to 34.4$\pm$5.5% of control ($P<0.001$, $n=6$; Figure 3B). The residual release represents a nonspecific efflux of [^{3}H]-dopamine: it is of similar magnitide to the release of radioactivity provoked by a buffer pulse or vehicle (0.05% DMSO, Figure 3B). Mecamylamine significantly decreased AnTx-evoked release in the presence of PDBu, from 208.3$\pm$14.2% to 94.9$\pm$10.1% of control ($P<0.05$, $n=6$), that is, mecamylamine decreased release to the level evoked by PDBu alone (101.4$\pm$8.3% of control, $n=6$, Figure 3B). Thus specific AnTx-evoked [^{3}H]-dopamine release,

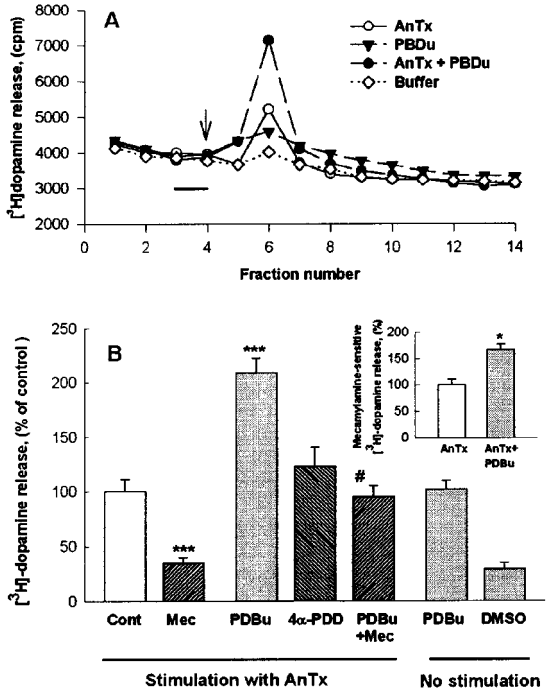

Figure 3 Effects of phorbol esters on AnTx-evoked [^{3}H]-dopamine release from superfused striatal synaptosomes. (A) Typical profiles for [^{3}H]-dopamine release from superfused striatal synaptosomes. Where used, PDBu (1 μM) was present for 2 min (bar) immediately preceding a 40 s stimulation (arrow) with AnTx (1 μM) or buffer. Fractions were collected at 2 min intervals and counted for released [^{3}H]-dopamine. (B) Synaptosomes were pretreated for 2 min with PDBu (1 μM), inactive 4α-PDD (1 μM) or vehicle DMSO (0.05%), prior to stimulation with AnTx (1 μM, 40 s) or buffer (no stimulation), in the continuous presence or absence of 10 μM mecamylamine (Mec). Inset: the potentiation by PDBu (1 μM) of specific [^{3}H]-dopamine release from synaptosomes (defined as the portion sensitive to mecamylamine and hence mediated *via* nicotinic ACh receptors). Values are the mean$\pm$s.e.mean of 6–9 experiments. Significantly different from corresponding controls: ***$P<0.001$, *$P<0.05$, Student's paired *t*-test; #$P<0.05$, one-way ANOVA, Tukey test.

calculated as the mecamylamine-sensitive portion of evoked release, was significantly increased (by 70–80% of control) in the presence of PDBu (Figure 3B, inset; Table 1).

The effects of PKC inhibitors on this potentiation of AnTx-evoked [^{3}H]-dopamine release by 1 μM PDBu were studied, to establish if PKC is involved. Both Ro 31-8220 (1 μM) and D-*erythro*-sphingosine (10 μM) significantly decreased mecamylamine-sensitive AnTx-evoked release in the presence and absence of PDBu, whereas the inactive bisindolylmaleimide V (1 μM) was without effect (Table 1).

In contrast, neither PDBu (1 μM) nor Ro 31-8220 (1 μM) modified significantly 15 mM KCl-stimulated [^{3}H]-dopamine release from synaptosomes when applied separately or together (data not shown). A low concentration of KCl was used to provoke the release of a similar amount of tritium as that achieved with 1 μM AnTx (see Soliakov *et al*., 1995).

Ca^{2+}-dependence of the potentiation by phorbol esters of AnTx-evoked and basal [^{3}H]-dopamine release from synaptosomes

The Ca^{2+} dependence of the actions of phorbol esters was examined by comparing basal and AnTx-evoked [^{3}H]-

Table 1 Effects of PKC activators and inhibitors on mecamylamine-sensitive release of [^{3}H]-dopamine from rat striatal synaptosomes stimulated with AnTx

Experimental conditions	*AnTx-evoked [^{3}H]-dopamine release (mecamylamine-sensitive fraction), % of control*
AnTx (1 μM), control	100.0 $\pm$ 8.0 (n = 24)
AnTx (1 μM) + Ro 31-8220 (1 μM)	**49.0 $\pm$ 4.6 (n = 8)
AnTx (1 μM) + D-*erythro*-Sphingosine (10 μM)	**73.9 $\pm$ 3.3 (n = 6)
AnTx (1 μM) + Bisindolylmaleimide V (1 μM)	106.6 $\pm$ 6.7 (n = 5)
AnTx (1 μM) + PDBu (1 μM)	***179.1 $\pm$ 13.6 (n = 16)
AnTx (1 μM) + PDBu (1 μM) + Ro 31-8220 (1 μM)	#61.1 $\pm$ 14.3 (n = 5)
AnTx (1 μM) + PDBu (1 μM) + D-*erythro*-Sphingosine (10 μM)	#112.2 $\pm$ 5.3 (n = 6)
AnTx (1 μM) + PDBu (1 μM) + Bisindolylmaleimide V (1 μM)	194.5 $\pm$ 9.4 (n = 5)

Significantly different from control: **P < 0.01; ***P < 0.001, Student's paired t-test. Significantly different from [^{3}H]-dopamine release evoked by AnTx (1 μM) + PDBu (1 μM): #P < 0.05, one-way ANOVA, Tukey test.

dopamine release, in the presence or absence of either PDBu or PMA, in normal and Ca^{2+}-free conditions (see Methods for details). In the presence of Ca^{2+}, PDBu and PMA (but not the inactive analogue 4α-PDD) potentiated the basal release of [^{3}H]-dopamine and this effect was not blocked by Ro 31-8220 (Figure 4A). There was a small but significant decrease in the stimulatory effects of PDBu and PMA on basal release in Ca^{2+}-free buffer, compared with normal Krebs-bicarbonate buffer. However, AnTx-evoked [^{3}H]-dopamine release was almost completely abolished in Ca^{2+}-free medium (to 10.8 $\pm$ 5.1% of control, n = 6; Figure 4B). Following treatment with PDBu (1 μM) or PMA (1 μM), AnTx-evoked release was markedly lower in the absence of Ca^{2+}, compared with the response in normal Krebs-bicarbonate buffer (Figure 4B), and the residual release corresponds very closely to that provoked by the phorbol esters in the absence of AnTx (Figure 4A). Thus the potentiation of nAChR-mediated [^{3}H]-dopamine release by phorbol esters appears to be Ca^{2+}-dependent.

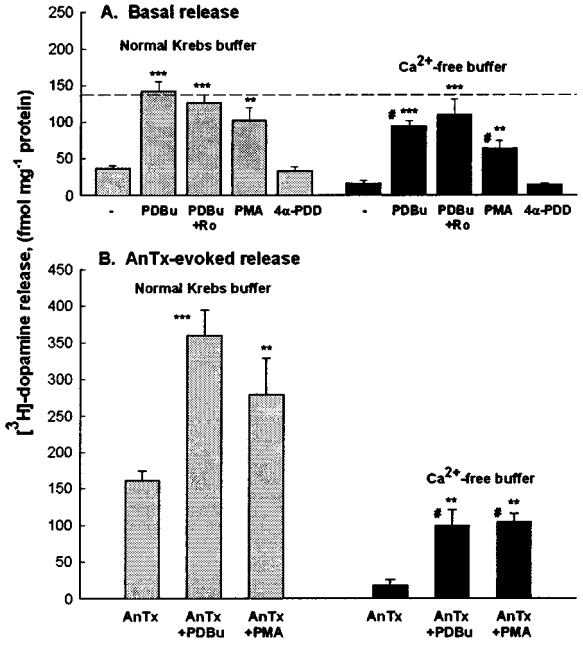

Figure 4 Ca^{2+}-dependence of the potentiation by phorbol esters of basal (A) and AnTx-evoked (B) [^{3}H]-dopamine release from rat striatal synaptosomes. (A) Synaptosomes were superfused with normal or Ca^{2+}-free medium containing EGTA in the presence or absence of Ro 31-8220 (Ro, 1 μM). Where indicated, synaptosomes were exposed to PDBu (1 μM), PMA (1 μM) or 4α-PDD (1 μM) for 2 min, followed by continuous perfusion in normal or Ca^{2+}-free medium. The dashed line indicates the release evoked by AnTx in normal Krebs-bicarbonate medium. (B) Synaptosomes were superfused as in (A), except that [^{3}H]-dopamine release was evoked by stimulation with AnTx (1 μM, 40 s) after pretreatment with phorbol ester (or buffer). Active phorbol esters significantly increased both basal and evoked release, in normal and in Ca^{2+}-free conditions. **P < 0.01; ***P < 0.001, Student's paired t-test, n = 4–6. The effect of phorbol esters in the Ca^{2+}-free condition was significantly less than in the corresponding condition in normal buffer (#P < 0.05, one-way ANOVA, Tukey test).

Discussion

In this study we began an examination of the interaction of presynaptic nicotinic ACh receptors with second messenger systems, here focusing on PKC. Several lines of evidence have been presented which indicate an involvement of PKC in nicotinic ACh receptor-mediated [^{3}H]-dopamine release from rat striatal synaptosomes. Two potent and specific PKC inhibitors, Ro 31-8220 and D-*erythro*-sphingosine, partially but significantly inhibited AnTx-evoked [^{3}H]-dopamine release, while two active phorbol esters significantly increased AnTx-evoked release of [^{3}H]-dopamine. The potentiation by phorbol esters was Ca^{2+}-dependent and reduced by PKC inhibitors.

The ability of two structurally and functionally unrelated PKC inhibitors to diminish AnTx-evoked [^{3}H]-dopamine release (Figure 1), coupled with the lack of effect of bisindolylmaleimide V (an inactive structural analogue of Ro 31-8220) is compelling evidence for the involvement of PKC, rather than a non-specific action such as antagonism of the nicotinic ACh receptor itself. The lack of an effect of Ro 31-8220 on nicotinic ACh receptors was confirmed by $^{86}Rb^+$ efflux experiments which monitor receptor function directly (Marks *et al*., 1996; Sharples *et al*., 2000; Figure 2). One interpretation is that agonist activation of nicotinic ACh receptors leads to the subsequent activation of PKC, resulting in increased exocytosis. This would be consistent with studies on chromaffin cells, in which the stimulation of catecholamine secretion by nicotine was modulated by drugs acting on PKC (TerBush *et al*., 1988; Vitale *et al*., 1992; Cox & Parsons, 1997). Inhibition of PKC has also been found to markedly attenuate nicotine-evoked increases in intracellular calcium in HEK 293 cells heterologously

expressing rat $\alpha4\beta2$ nicotinic ACh receptors (Eilers *et al*., 1997).

The mechanism by which nicotinic ACh receptors may lead to the activation of PKC is not clear but has also been proposed to occur with other experimental systems (Eusebi *et al*., 1987; Nishizaki & Sumikawa, 1998a). Attempts to demonstrate translocation of PKC to the membrane in striatal synaptosomes, in response to AnTx stimulation, were unsuccessful (L. Soliakov and S. Wonnacott, unpublished data). This negative result may reflect the heterogeneity of the preparation, with nicotinic ACh receptors present on only a minority of synaptosomes: dopamine nerve terminals constitute approximately 10% of the population of striatal synaptosomes (Wolf & Kapatos, 1989). Alternatively, translocation of PKC may not be a prerequisite for activation: enzyme already resident in membranes may be activatable (Chakravarthy *et al*., 1994). Thus membrane-bound PKC, perhaps in the vicinity of the nicotinic ACh receptor where it may be activated by Ca^{2+} entering through the nicotinic channel, may mediate the observed enhancement of [^{3}H]-dopamine release.

In addition to the Ro 31-8220-sensitive component of AnTx-evoked [^{3}H]-dopamine release, a short exposure to phorbol esters was found to enhance mecamylamine-sensitive AnTx-evoked [^{3}H]-dopamine release, implying that nicotinic ACh receptor activation and independent PKC activation have a synergistic effect on transmitter release. This process was complicated by the elevation of basal release by phorbol esters: although this was not seen with the inactive phorbol ester 4α-PDD, it was not prevented by Ro 31-8220 and was largely Ca^{2+}-independent (Figure 4). There are numerous reports of the ability of phorbol esters to provoke transmitter release in the absence of other stimulating agents (Dekker *et al*., 1991; Waters & Smith, 2000), although this is not always considered when examining evoked release. One candidate for mediating the Ro 31-8220-insensitive increase in basal release is the novel presynaptic phorbol ester receptor Munc 13-1 (Betz *et al*., 1998). This protein is proposed to enhance transmitter release in a phorbol ester-dependent, PKC- and Ca^{2+}-independent manner by interacting with the docking protein DOC2, resulting in its translocation from vesicles to plasma membrane (Duncan *et al*., 1999), thus effecting a priming step in exocytosis (Augustin *et al*., 1999).

Taking the phorbol ester stimulation of basal release into account, PDBu increased mecamylamine-sensitive (and hence nicotinic ACh receptor-mediated) AnTx-evoked [^{3}H]-dopamine release by about 70–80% (Figure 3; Table 1). This increase was Ca^{2+}-dependent and prevented by PKC inhibitors (Figure 4, Table 1). Thus phorbol esters exert two effects on [^{3}H]-dopamine release in these experiments: a PKC-independent enhancement of basal release, and a PKC-dependent enhancement of nicotinic ACh receptor-evoked release. Despite the abundance of evidence for potentiation by PKC of evoked transmitter release (e.g. Nichols *et al*., 1987; Dekker *et al*., 1991; Coffey *et al*., 1993; Terrian, 1995; Waters & Smith, 2000), there is little consensus about its precise contribution. Mechanisms proposed include a direct effect on ion channels (Coffey *et al*., 1993) or an increase in the size of the readily releasable pool of vesicles (Waters & Smith, 2000). In chromaffin cells, enhancement of the nicotinic secretion of catecholamines by short term phorbol ester treatment has been attributed to disruption of the actin cytoskeleton (Vitale *et al*., 1995; Trifaro *et al*., 2000) and potentiation of an ATP-dependent priming step by phosphorylation of GAP-43 (Misonou *et al*, 1998).

Another possibility is that PKC may specifically enhance AnTx-evoked [^{3}H]-dopamine release through a direct effect on the nicotinic ACh receptor: rat $\alpha3$, $\alpha4$ and $\beta2$ subunits (implicated in the presynaptic nicotinic ACh receptors modulating striatal dopamine release; Sharples *et al*., 2000) have consensus sites for PKC phosphorylation (Goldman *et al*., 1987; Fenster *et al*., 1999). Phosphorylation of neuronal ACh receptors has been reported to increase channel conductance (Nishizaki & Sumikawa, 1998b) and increase the rate of recovery from desensitization (Fenster *et al*., 1999). Thus receptor phosphorylation would be compatible with the increased nicotinic responses measured here.

In conclusion, this study provides clear evidence that a component of AnTx-evoked [^{3}H]-dopamine release from rat striatal synaptosomes is mediated by PKC. In addition, independent activation of PKC (in this case by phorbol esters) potentiated AnTx-evoked [^{3}H]-dopamine release. The latter observation could facilitate crosstalk between metabotropic receptors coupled to PKC activation and nicotinic ACh receptor responses.

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